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# Induction of V3-Specific Cytotoxic T Lymphocyte Responses by HIV *gag* Particles Carrying Multiple Immunodominant V3 Epitopes of gp120

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Efforts to develop a vaccine to prevent infection of human immunodeficiency virus (HIV) have focused on the induction of neutralizing antibodies. In our previous study, we reported that chimeric *gag–env* virus-like particles (VLPs) induce neutralizing antibodies which block HIV infection. In addition to the neutralizing antibodies, the cytotoxic T-lymphocyte (CTL) response is considered to be another major immune defense mechanism required for recovery from many different viral infections. In the present study, we have constructed chimeric fusion proteins using HIV-2 *gag* precursor protein with (1) four neutralizing epitopes from HIV-1 gp160; (2) three tandem copies of consensus V3 domain, which have been derived from 245 different isolates of HIV-1 and carries both the principal neutralizing determinant (PND) and CTL epitopes; and (3) V3 domains from HIV-$1_{IIIB}$, HIV-$1_{MN}$, HIV-$1_{RF}$, and HIV-$1_{SF2}$. These chimeric fusion proteins were expressed in a large quantity within insect cells, and released as VLPs into the cell culture medium. The purified *gag–env* VLPs from all three constructs appear to be spherical particles similar to immature HIV but slightly larger than the *gag* VLPs. Immunoprecipitation analysis showed that the chimeric proteins were recognized not only by HIV-1 positive patient sera, but also by monoclonal and polyclonal antisera raised against V3 peptides of HIV-$1_{IIIB}$, HIV-$1_{MN}$, HIV-$1_{RF}$, and the gp120 antiserum against HIV-$1_{SF2}$. Balb/C mice immunized with these chimeric VLPs successfully induced CTL activity against V3 peptide-stimulated target cells. In addition, a high degree of cross-reactivity was observed among the four different strains of HIV-1 V3 domain, indicating that the tandem multiple consensus V3 peptide sequence carried by HIV-2 *gag* can be used as a potential HIV vaccine against various HIVs. 

## INTRODUCTION

The development of an AIDS vaccine to prevent HIV infection has been focused on the induction of both humoral and cell mediated immune responses. The neutralizing antibodies eliminate extracellular viruses, whereas the viruses primarily spread by virus-infected cells which can be destroyed by the viral-specific cytotoxic T lymphocytes (CTLs)(Townsend *et al.,* 1986; Rouse *et al.,* 1988; Klavinskis *et al.,* 1989). The CTLs that are capable of lysing target cells expressing HIV proteins have been detected in HIV-infected patients, and these HIV-specific CTLs can inhibit HIV replication in peripheral blood lymphocytes (Tsubota *et al.,* 1989). Accordingly, CTLs appear to play a critical role in the control of HIV infection.

HIV-1 V3 domain has been shown to be an immunodominant site of principal neutralizing determinant (PND) and contains the T-cell epitopes of the HIV *env* glycoprotein. Therefore, the V3 region contains an important sequence for a vaccine formulation (Berzofsky *et al.,* 1991; Luo *et al.,* 1992; Casement *et al.,* 1995). However, neutralizing antibodies found in HIV-1-infected individuals, or induced by immunizing animals with *gag*-V3 chimeric proteins derived from a single strain of HIV-1, or induced by candidate HIV-1 vaccines based on gp120 have been predominantly type specific, thus indicating their limited value as an AIDS vaccine (Goudsmit *et al.,* 1988; Myers 1989). The induction of a broadly reactive immunity against HIV has been impaired by the extent of sequence variations exhibited by variants of the HIV-1 isolate. One particularly disturbing finding is that there is a high degree of antigenic variation in gp160, showing at least 25% of the amino acids differ. The V3 domain is one of the more variable regions of gp120 and differs by as much as 50% among HIV-1 isolates (Nara *et al.,* 1988).

In the previous study, we introduced neutralization epitopes from gp120 into the carrier protein HIV-2 *gag,* which is an effective vector for the delivery and expression of foreign genes in a baculovirus system, and presented the antigen as a polyvalent particulate structure (Luo *et al.,* 1992). In the present study, we have also employed the HIV-2 *gag* as carrier and constructed chimeric genes by coupling the HIV-2 *gag* gene with four different epitopes from HIV-1 gp160, with three tandem

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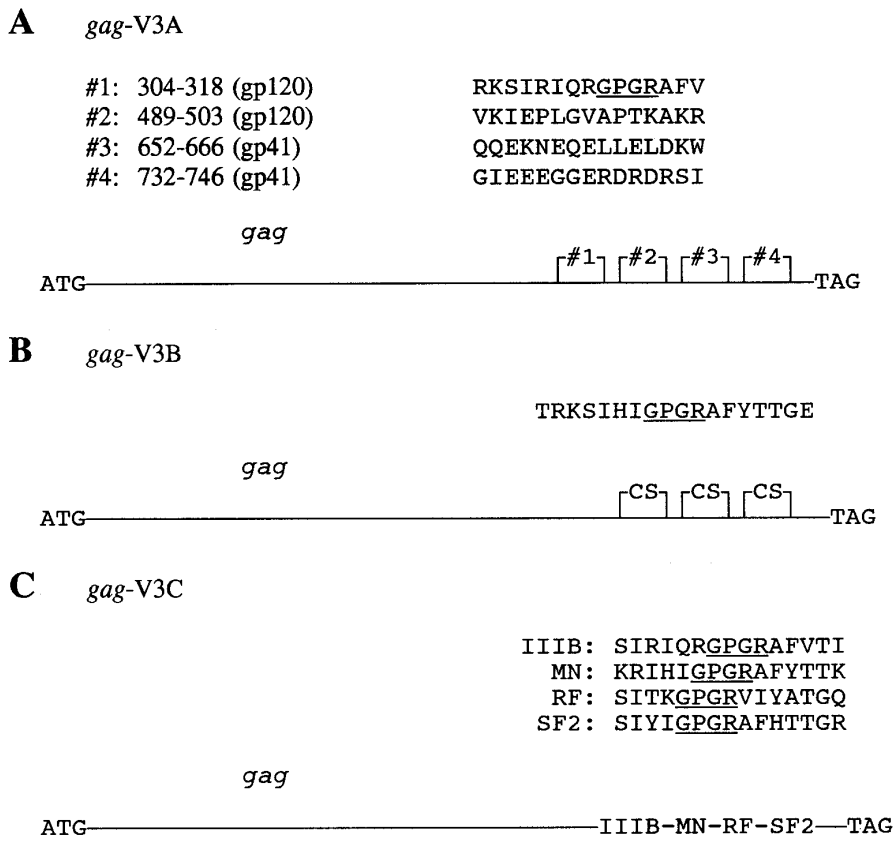


FIG. 1 Schematic representation of three chimeric *gag–env* genes containing multiple neutralizing epitopes of HIV-1 The three sets of synthetic *Bgl*II-digested DNA fragments, which contain four different neutralizing epitopes of HIV-1 gp160, two from gp120 and two from gp41 (A), three tandem copies of consensus V3 sequence obtained from 245 different HIV-1 isolates (B), and multiple V3 sequences from HIV-$1_{IIIB}$, HIV-$1_{MN}$, HIV-$1_{RF}$, and HIV-$1_{SF2}$ (C) were phosphorylated and inserted into the *Bgl*II site of the C-terminus of truncated HIV-2 *gag* gene, which is just in front of the stop codon TAA of *gag* gene (Luo *et al.,* 1990, 1992). The synthetic amino acid sequences and the order of the multiple neutralizing epitopes are indicated.

copies of consensus V3 loop sequence ($V3_{CS}$) from 245 isolates of HIV-1 or with multiple V3 loop sequences from IIIB, MN, SF2, and RF strains of HIV-1 It has been demonstrated that subunit vaccines containing multiple copies of Vp1 protein of foot-and-mouth disease virus (Brown *et al.,* 1988) and of gD protein of herpes simplex virus (Prevec and Rosenthal, McMaster University, pers. commun.) showed strong immune responses.

Large quantities of *gag–env* fusion proteins have been produced in insect cells using a baculovirus expression system and used to evaluate the ability to induce both humoral and cellular immune responses.

## RESULTS

### Construction and expression of chimeric proteins

In an attempt to produce *gag–env* chimeric proteins for eliciting both humoral and cell mediated immune responses, a subclone of the truncated HIV-2$gag_{425}$ gene in the pUC19 plasmid was used as a carrier of the multiple V3 genes to form *gag–env* chimeric proteins. The basic strategy used to construct plasmids for expression of *gag*–V3 fusion proteins is illustrated in Fig. 1 and described under Materials and Methods. The four different oligonucleotide sequences from fragments of HIV-1 gp160, which were postulated to be antigenic epitopes (Broliden *et al.,* 1992), the three tandem copies of the consensus principal neutralizing determinant (PND), which were based on the V3 loop consensus sequence obtained from 245 HIV-1 isolates (LaRosa *et al.,* 1990), and multiples PNDs from four different isolates, HIV-$1_{IIIB}$, HIV-$1_{MN}$, HIV-$1_{RF}$, and HIV-$1_{SF2}$ (Takahashi *et al.,* 1992), were synthesized individually as indicated in Fig. 1 and fused directly onto the C-terminal *Bgl*II site of pUC19-GAG of HIV-2. The resulting plasmids were digested with the restriction enzyme *Bam*HI and subcloned into the baculovirus transfer vector pBacPAK1 Following cotransfection with wild type BacPAK6 DNA, three corresponding recombinant baculoviruses were generated and designated as Ac–*gag*–V3A, Ac–*gag*–V3B, and Ac–*gag*–V3C. The ability of each of the recombinant baculoviruses to direct the synthesis of the *gag*–V3 fusion protein was tested in SF21 cells. The whole cell lysates were prepared at 3 days (postinfection) and analyzed by SDS–PAGE and Western blot. By Coomassie blue staining of proteins from cell lysates, we

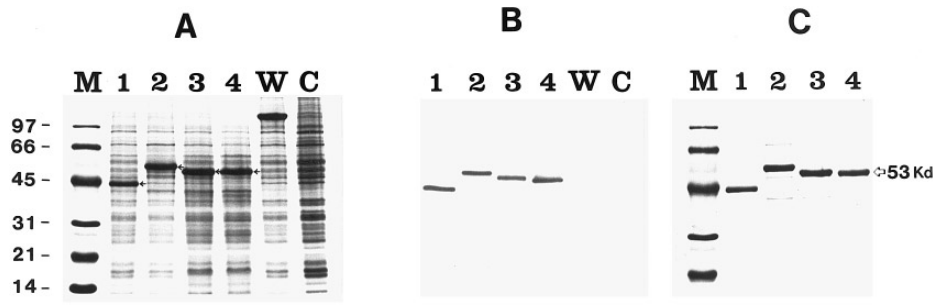


FIG. 2. Expression of chimeric *gag–env* proteins in SF21 cells infected by recombinant baculoviruses. Infected cells were harvested at 3 days p.i. and whole cell lysates were analyzed by 12% SDS–PAGE stained with Coomassie blue (A) and detected by Western blot (B) using HIV positive patient serum. Lanes 1–4: recombinant viruses AcNPV-HIV-2*gag*-, Ac–*gag*–V3A-, Ac–*gag*–V3B-, and Ac–*gag*–V3C-infected cells. The chimeric *gag* particles purified from the culture supernatant were also subjected to SDS–PAGE and stained with Coomassie blue (C). M, marker protein; C, uninfected cell control; Wt, wild-type BacPAK6-infected cells. The major fusion protein P53 is indicated with an arrow. The positions of molecular standard are given in kDa.

observed both *gag* protein alone and chimeric *gag* proteins produced by recombinant baculoviruses AcNPV-HIV-2*gag* (Luo *et al.,* 1990), Ac–*gag*–V3A, Ac–*gag*–V3B, and Ac–*gag*–V3C (Fig. 2A, lanes, 1, 2, 3, and 4) but were not present in those of mock or wild type baculovirus-infected cells. Since the original HIV-2*gag* protein has 94 amino acids deleted from the C-terminus (Luo *et al.,* 1990), an insertion of multiple antigenic epitopes of HIV-1 gp160 (60 amino acids), three tandem copies of consensus V3 sequence (54 amino acids), or multiple V3 sequence (60 amino acids) from four different isolates of HIV-1 into the C-terminus of *gag* gene would be expected to produce a protein of approximately 54 kDa for *gag*–V3A and *gag*–V3C and approximately 53 kDa for *gag*–V3B. As shown in Fig. 2A, strongly stained protein band migration at either 54 or 53 kDa were observed in the lysates of SF21 cells infected with Ac–*gag*–V3A (lane 2) and Ac–*gag*–V3B (lane 3). However, the fusion protein *gag*–V3C expressed by Ac–*gag*–V3C differed slightly in its mobility from *gag*–V3A, although the same size V3 sequences were added in both constructs (lane 4). This accelerated migration could be due to the different conformation of chimeric protein when V3A and V3C were fused with *gag.* An alternative explanation is the charge differences between the amino acids in V3A and V3C. We have observed this kind of variation in other proteins although we have used SDS in the gel (unpublished observation). Western blot analysis further revealed that both 54 and 53-kDa fusion proteins were recognized by pooled HIV-1 positive human sera. No specific reaction was observed with mock and wild type baculovirus-infected cells (Fig. 2B).

## The assembly of *gag–env* chimeric protein into spherical virus-like particles

We next asked whether the chimeric *gag–env* proteins were able to assemble virus-like particles (VLPs) and secreted into the cell culture media. To this end, each of the three recombinant baculovirus-infected cell culture supernatants, including AcNPV-HIV-2*gag* as positive control, were harvested at 2 days p.i. before significant cell lysis had occurred. After ultracentrifugation of culture supernatants, the pellet was resuspended and layered on a 20–60% discontinuous sucrose gradient as described previously (Luo *et al.,* 1990, 1992). We found that all of the three recombinant baculoviruses, Ac–*gag*–V3A, Ac–*gag*–V3B, and Ac–*gag*–V3C, produced sedimentable chimeric virus-like particles, as did the original *gag* protein alone. Figure 2C shows the purified HIV-2*gag* particles (lane 1), chimeric particles *gag*–V3A (lane 2), *gag*–V3B (lane 3), and *gag*–V3C (lane 4) analyzed on SDS–PAGE and visualized by Coomassie blue staining. The size of the major fusion protein is identical to that of the fusion protein found in cell lysate (Fig. 2A) and further confirmed by Western blot (data not shown). These results demonstrated that the fusion proteins had been packaged into VLPs.

To analyze the morphology of the chimeric *gag–env* VLPs, the sucrose gradient purified chimeric VLPs were examined by transmission electron microscopy (EM) using uranyl acetate staining. The electron micrograph of chimeric VLPs and the HIV-2*gag* particles without fusion with *env* domain is shown in Fig. 3. As we reported previously, these spherical particles had a diameter of 110–130 nm and were similar to that of mature HIV-1 particles budding from HIV-1 infected cells (Gelderblom *et al.,* 1987; Luo *et al.,* 1990). The chimeric VLPs, *gag*–V3A (Fig. 3B), *gag*–V3B (Fig. 3C), and *gag*–V3C (Fig. 3D), appear to have similar morphology but slightly larger than that of the *gag* particles without fusion with other amino acids (Fig. 3A).

## Antigenic characterization of V3 domain fused in *gag* particles

To determine whether the three different *gag*–V3 fusion particles can be recognized by the V3-specific sera, raised against peptides of the V3 domain of HIV-$1_{IIIB}$, HIV-$1_{MN}$, HIV-$1_{RF}$, or HIV-$1_{SF2}$, the antigenicity of the chi-

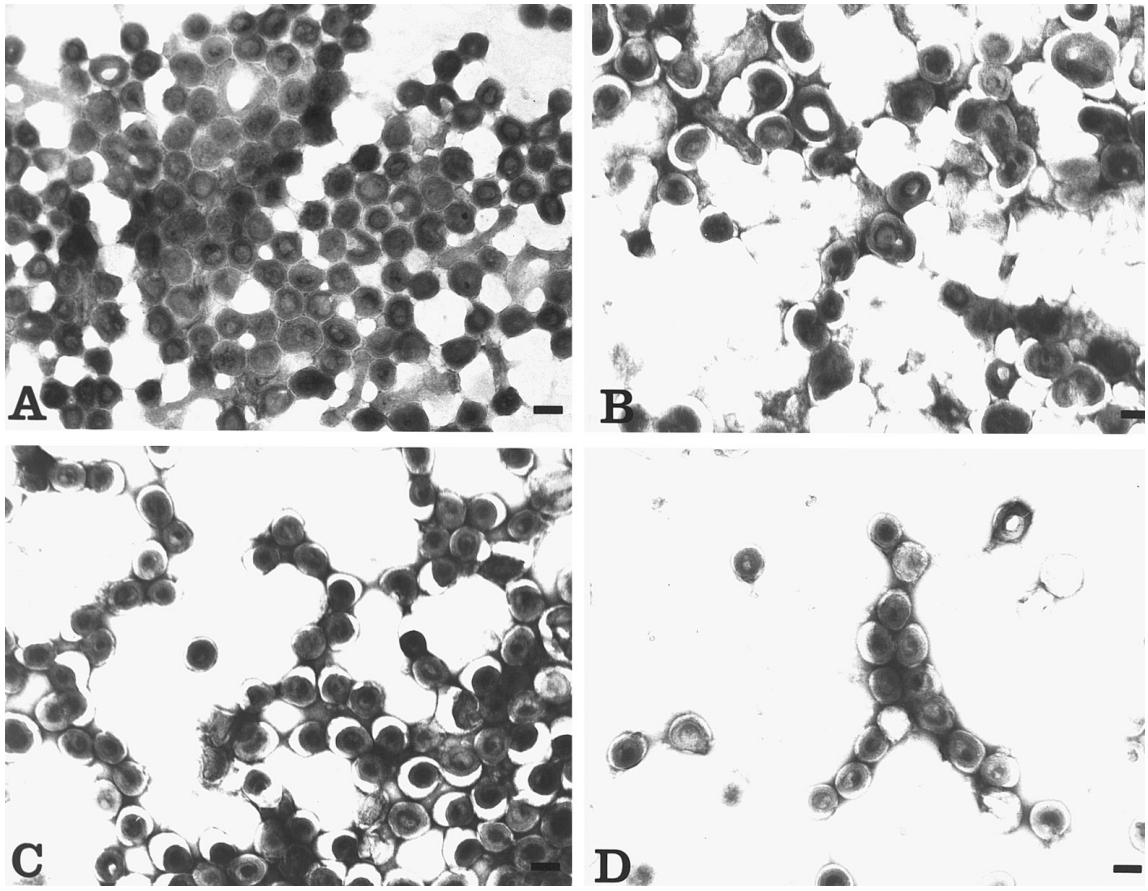


FIG. 3. Electron micrograph of sucrose gradient-purified *gag–env* chimeric particles stained with uranyl acetate. (A) HIV-2 *gag* particles produced by SF21 cells infected with recombinant AcNPV-HIV-2*gag* virus as control. (B) Chimeric *gag* particles produced by recombinant Ac–*gag*–V3A. (C) Chimeric *gag* particles produced by recombinant Ac–*gag*C–V3B. (D) Chimeric *gag* particles produced by recombinant Ac–*gag*–V3C. Bar represents 100 nm.

meric proteins was examined by pulse labeling the infected cells with [$^{35}$S]methionine for 1 h at 24 h p.i. and immunoprecipitated with a panel of antisera and analyzed in SDS–PAGE. Figure 4 shows that the cell lysates from SF21 cells infected with AcNPV-HIV-2*gag* (lane 1), Ac–*gag*–V3A (lane 2), Ac–*gag*–V3B (lane 3), and Ac–*gag*–

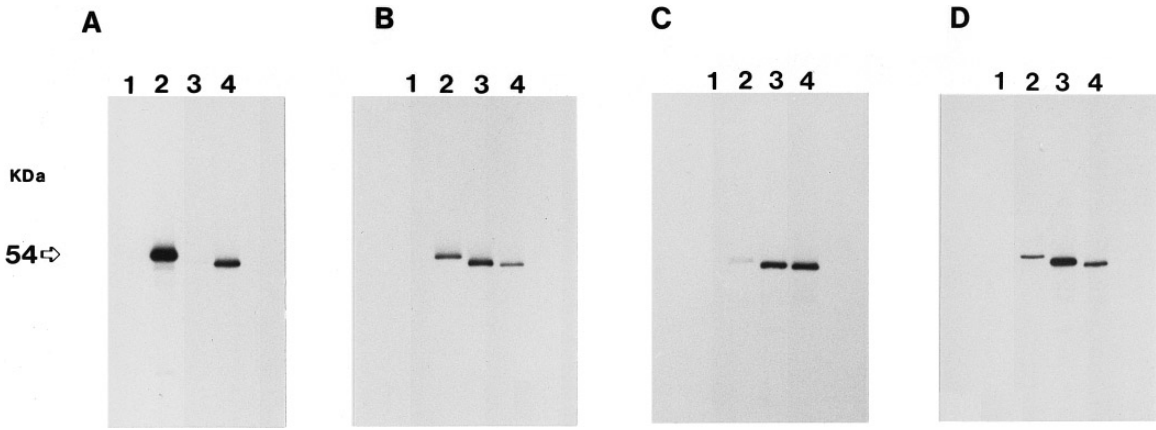


FIG. 4. Analysis of antigenicity of *gag*–V3 chimeric protein. SF21 cells were infected at m.o.i. of 5 PFU/cell with each recombinant baculoviruses expressing the truncated *gag* or the *gag*–V3 chimeric proteins. After 24 h p.i. cells were pulse-labeled for 1 h with [$^{35}$S]methionine. The cells infected with recombinant viruses AcNPV-HIV-2*gag,* Ac–*gag*–V3A, Ac–*gag*–V3B, and Ac–*gag*–V3C were harvested separately and equal amounts were used for immunoprecipitation with anti-$V3_{IIIB}$ monoclonal antibody (A), anti-$V3_{MN}$ antiserum (B), anti-$V3_{RF}$ antiserum (C), and anti-$gp120_{SF2}$ antiserum (D). The immunoprecipitated samples were analyzed in 12% SDS–PAGE and visualized by fluorography. Lane 1, recombinant viruses AcNPV-HIV-2*gag;* lane 2, Ac–*gag*–V3A; lane 3, Ac–*gag*–V3B; lane 4, Ac–*gag*–V3C-infected cells. The empty arrow indicates the chimeric protein size of Ac–*gag*–V3A.

TABLE 1A

Anti-*gag* Particle Responses

| Antiserum | Anti-*gag* | Anti-*gag*-V3A | Anti-*gag*-V3B | Anti-*gag*-V3C |
|---|---|---|---|---|
| *gag* particles | 2.17 | 1.09 | 0.98 | 1.52 |

*Note.* Data represent $A_{415}$ values at 1:51,200 dilutions. Concentration of *gag* particles: 100 ng/well.

TABLE 1B

Anti-V3 Immune Response Induced by Recombinant Chimeric Particles

| Chimeric particles serum (dilutions) | V3 peptides | Absorbance (415 nm) |
|---|---|---|
| Anti-*gag*–V3A (1:1000) | V3A | 0.11 |
| Anti-*gag*–V3B (1:100) | V3B | 0.12 |
| Anti-*gag*–V3C (1:6400) | V3C | 0.21 |

*Note.* Concentration of V3 peptides: 100 ng/well.

V3C (lane 4) were immunoprecipitated separately with anti-$V3_{IIIB}$ monoclonal antibody (MAb) (Fig. 4A), anti-$V3_{MN}$ MAb (Fig. 4B), anti-$V3_{RF}$ MAb (Fig. 4C), and anti-$gp120_{SF2}$ from purified $gp120_{SF2}$ (Fig. 4D). As expected, the HIV-2 *gag* protein expressed without *env* fusion by AcNPV-HIV-2*gag* recombinant virus (Luo *et al.,* 1990) was neither recognized by antibodies against $V3_{IIIB}$, $V3_{MN}$, or $V3_{RF}$ peptides nor by antibody against $gp120_{SF2}$ (Figs. 4A, 4B, 4C, and 4D, lane 1). In contrast, the *gag*–V3A, *gag*–V3B, and *gag*–V3C fusion proteins showed a strong reactivity with their corresponding antisera against $V3_{IIIB}$, $V3_{MN}$, $V3_{RF}$ or $gp120_{SF2}$ (Fig. 4A, lane 2; 4B, lane 3; 4C and 4D, lanes 4). The HIV-$1_{IIIB}$ V3-specific MAb showed a strong reactivity only with *gag*–V3A and *gag*–V3C chimeric proteins (Fig. 4A, lanes 2 and 4) which contained HIV-$1_{IIIB}$ V3 sequence, but barely binding with *gag*–V3B (Fig. 4A, lane 3). Although the *gag*–V3B contained three tandem copies of consensus sequences of V3 domains of some 245 HIV-1 isolates, this sequence $V3_{CS}$ is closely related to HIV-$1_{MN}$ strain and has 87% homology sequences with $V3_{MN}$ (Fig. 1B). To determine the antigenicity of *gag*–V3B, we employed the anti-$V3_{MN}$ antiserum to test whether it recognizes the inserted $V3_{CS}$ sequence. As expected, the anti-$V3_{MN}$ antibodies exhibited not only strong binding to *gag*–V3B (Fig.4B, lane 3) but also to *gag*–V3A and *gag*–V3C (Fig. 4B, lanes 2 and 4). In the same manner, both the anti-$V3_{RF}$ and anti-$gp120_{SF2}$ antibodies also recognized *gag*–V3C, which contained $V3_{IIIB}$, $V3_{MN}$, $V3_{RF}$, and $V3_{SF2}$ sequence, or bound to *gag*–V3A and *gag*–V3B chimeric proteins (Figs. 4C and 4D). The monoclonal antibody against V3 peptides of HIV-$1_{IIIB}$ strain reacted only with $V3_{IIIB}$. This result is consistent with previous reports (Rusche *et al.,* 1988; Javaherian *et al.,* 1989). These results clearly demonstrate that the chimeric proteins can be detected by anti-V3 antibodies, which reaffirm the evidence that inserted sequences of multiple V3 epitopes are antigenic and indicate the cross-reactivity between anti-$V3_{MN}$, anti-$V3_{RF}$, and anti-$gp120_{SF2}$ antisera.

## Immune responses to *gag–env* fusion VLPs

In order to examine whether the *gag–env* chimeric particles are capable of inducing antibody response to both *gag* and inserted peptides of *env* protein, each group of four rabbits were immunized four times at 4-week intervals with purified *gag*–V3A, *gag*–V3B, or *gag*–V3C chimeric particles as described under Materials and Methods. The immune rabbit sera, obtained 2 weeks after the last boost immunization, and preimmune sera were tested for their ability to recognize *gag* protein and V3 peptides from *gag*–V3A, *gag*–V3B, and *gag*–V3C anti-*gag* and anti-V3 antibodies levels were assessed by ELISA. All rabbit groups generated a strong anti-*gag* response (Table 1A), demonstrating that the chimeric *gag*–V3 VLPs are potent immunogens. The *gag*–V3A, *gag*–V3B, and *gag*–V3C VLPs induced anti-V3 responses of 1:1000, 1:100, and 1:6400, respectively (Table 1B). This value is relatively higher than the previously published data (Truong *et al.,* 1995). Consistent with these results, approximately $\frac{1}{10}$ dilution of antiserum was necessary to inhibit 50% of homologous HIV-1 replication (data not shown). Similar results have been reported by other investigators. (Griffiths *et al.,* 1993; Truong *et al.,* 1995; Wagner *et al.,* 1996), suggesting that the weak neutralizing activity might be due either to an improper folding or to limited accessibility of the inserted epitopes.

## Induction of V3 peptide-specific CTLs with *gag*–V3C chimeric particles

To test *gag*–V3C chimeric particles representing multiple V3 sequences from IIIB, MN, RF, and SF2 strains of HIV-1 for induction of V3 peptide-specific CTL responses *in vivo,* the Balb/C mice were immunized with 50 μg of purified *gag*–V3C chimeric particles, spleens were removed from primed mice at 5 weeks postimmunization, and spleen cells were cultured. The pooled splenocytes were divided into four aliquots and restimulated *in vitro* with synthetic peptides of $V3_{IIIB}$, $V3_{MN}$, $V3_{RF}$, or $V3_{SF2}$. The resulting effector cells were tested for their ability to lyse the syngeneic target cells preincubated with cognate V3 peptides of IIIB, MN, RF, and SF2 strains of HIV-1. Figure 5 showed that the splenocytes from mice immunized with *gag*–V3C particles resulted in specific CTL responses for all four V3 peptide-stimulated target cells, but the CTLs induced by $V3_{RF}$ peptide exhibited a relatively low level of specific lysis of target cells at an E:T ratio of 5:1, 10:1, and 25:1. This result is consistent with a previous report by Casement *et al.* (1995) that V3 peptide from HIV-$1_{RF}$ is a weak inducer of CTLs. In contrast, V3 peptide-stimulated target cells were not lysed by effector cells primed by synthetic peptides from LCMV nucleo-

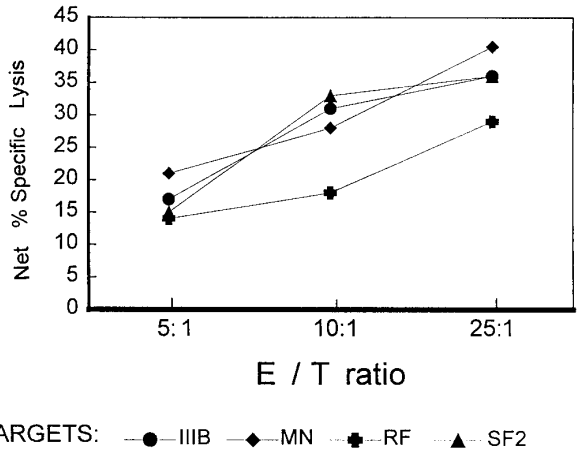


FIG. 5. Induction of CTLs in Balb/C mice by immunization with purified *gag*–V3C chimeric particles. Balb/C mice were primed and boosted once (at day 7) with V3C particles, corresponding to four different V3 sequences from IIIB, MN, RF, and SF2 strains of HIV-1 (Fig. 1C) in liposome emulsion (50 μg/100 μl/dose). Spleen cells from two mice per group were pooled and restimulated with $V3_{IIIB}$, $V3_{MN}$, $V3_{RF}$, or $V3_{SF2}$ peptides *in vitro* for 7 days. CTL activity was measured using MHC-matched p815 target cells, which were pretreated with $V3_{IIIB}$, $V3_{MN}$, $V3_{RF}$, or $V3_{SF2}$ peptides, at effector to target cell ratios (E:T) of 5:1, 10:1, and 25:1 (see Materials and Methods). The cytotoxic response was tested in a standard 6 h $^{51}$Cr-release assay. All levels of specific lysis are shown, in which nonspecific background has been subtracted.

capsid protein (NP) and the LCMV NP peptide-stimulated target cells were also not lysed by the effector cells primed by V3C or V3B particles (data not shown). These results indicated that the *gag*–V3 chimeric particle is capable of inducing V3 peptide-specific CTL responses *in vivo.*

## Induction of cross-reactivity of CTLs by consensus V3 sequence

Previous reports have demonstrated that the V3 domain is one of the more variable regions of the envelope glycoprotein and differs by as much as 50% in different HIV-1 isolates (Myers 1989; Nara *et al.,* 1988). For this reason, We have expressed *gag*–V3B chimeric particles which contain three tandem copies of consensus V3 sequence derived from 245 HIV-1 isolates, and tested whether it could induce a broadly cross-reactive CTLs against the variability of different isolates of HIV-1 As expected, the splenocytes from mice immunized with these purified chimeric *gag*–V3B particles developed a broad CTL response after *in vivo* and *in vitro* restimulation by $V3_{IIIB}$ peptides. Those splenocytes lysed the syngeneic target cells which were preincubated with individual cognate $V3_{IIIB}$, $V3_{MN}$, $V3_{RF}$, and $V3_{SF2}$ peptides. The cross-reactive lysis of P815 target cells in the range of 40–52% at an E:T ratio of 25:1 was observed (Fig. 6). This result is consistent with the previous report (Casement *et al.,* 1995) that the V3 peptides from HIV-$1_{MN}$ strain appear to be more efficient than others, inducing a broadly cross-reactive CTL response.

## Characterization of the CTLs induced by *gag*–V3B chimeric particles

Endogenously synthesized viral antigens are fragmented by antigen-processing enzymes in the cytoplasm or endoplasmic reticulum and presented on the surfaces of virus-infected cells in conjunction with class I major histocompatibility complex (MHC) molecules. Such processed peptides of viral proteins associated with class I molecules can be recognized by $CD8^+$ CTLs that kill the virus-infected cells. However, blocking of the antigen presenting MHC molecule with a specific antibody would inhibit target cell lysis. To determine whether the CTL effector function in splenocytes from immunized mice was due to $CD8^+$ or $CD4^+$ T cell, we treated the CTL effectors, induced by immunizing the mice with *gag*–V3B particles, with anti-CD8 or anti-CD4 antibody. The results shown in Fig. 7 demonstrate that the anti-CD8 serum treated CTL effectors abolished the cytotoxicity against IIIB peptide-stimulated p815 target cells. In contrast, pretreatment of effector cells with anti-CD4 antibody had no significant effect. Similar results were also obtained with CTLs generated in mice immunized with *gag*–V3C particles. These results further demonstrate that the cytotoxic response detected was mediated by class I-restricted $CD8^+$ CTL.

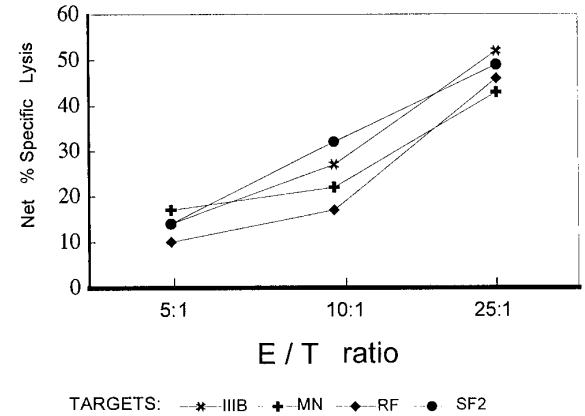


FIG. 6. Analysis of cross-reactivity of CTLs induced by *gag*–V3B purified chimeric particles. Balb/C mice were immunization and boosted once (at day 7) with *gag*–V3B chimeric VLPs, which contain three tandem copies of consensus V3 loop sequence (Fig. 1B), in liposome emulsion (50 μg/100 μl/dose). The pooled splenocytes from two mice per group were restimulated with $V3_{IIIB}$ peptide *in vitro* for 7 days, while the MHC-matched p815 target cells were pulsed with $V3_{IIIB}$, $V3_{MN}$, $V3_{RF}$, or $V3_{SF2}$ peptides. CTL activity of splenocytes from the immunized mice was measured at various effector and target cell ratios (E:T) of 5:1, 10:1, and 25:1 in a standard of 6 h $^{51}$Cr release assay as described under Materials and Methods. Specific lysis of target cells are shown with nonspecific background lysis subtracted.

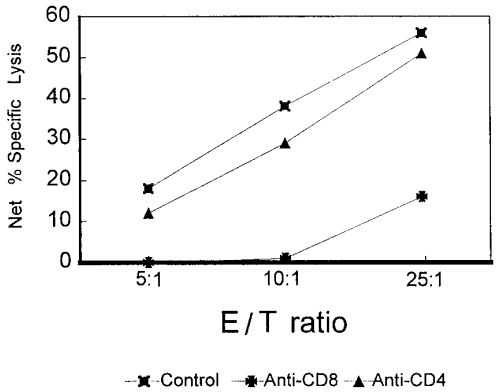


FIG. 7. The $CD8^+$ T cell subpopulation was responsible for the cytotoxic activity. The CTLs induced by immunization with *gag*–V3B chimeric particles containing three tandem copies of consensus V3 loop sequence were depleted mostly by preincubation with anti-CD8 antibody, but not with anti-CD4 antibody. The MHC-matched p815 target cells were stimulated with $V3_{IIIB}$ peptides. The ratio of the effector to target cell (E:T) are indicated on the bottom. The percentage of specific lysis is shown after subtracting the background lysis of control.

## DISCUSSION

In our present study we used truncated *gag* protein of HIV-2 (Luo *et al.,* 1990) to carry heterologous epitopes representing V3 domain of gp120 from various strains of HIV-1. In all three fusion constructs described in this paper, we have attached multiple V3 sequences at the C-terminus of the *gag* protein as shown in Fig. 1, which has the capacity to code for 54 or 60 additional amino acids, which is smaller in size than the deleted portion of the *gag* gene (Luo *et al.,* 1990). All three constructs representing HIV-1 multiple V3 sequence were efficiently packaged into VLPs that were morphologically similar to virus-like *gag* particles without additional fusion sequences. This result is consistent with our previous report that the C-terminus of HIV-2*gag* protein is not essential for VLPs formation and further demonstrates that by using the smaller size of heterologous proteins to substitute the deleted C-terminal portion of *gag* renders assembly and release of the chimeric fusion protein particles (Luo *et al.,* 1992).

In this report, we present the cytotoxic T cell induction by three intramuscular immunizations using *gag*–V3 VLPs entrapped in liposome. The V3 domain specific CTL response with broad reactivity patterns can be elicited by three tandem copies of consensus V3 peptide (*gag*–V3B) or multideterminant clusters of V3 peptide (*gag*–V3C) immunization. Our results show that the *gag*–V3 particles induced cytotoxic T-cell responses to a broad spectrum of HIV-1 clade B isolates. This is consistent with the report by Casement *et al.,* (1995) that the V3 peptide from MN strain of HIV-1 induced CTLs that exhibited cross-reactivity against a broad range of HIV-1 strains and suggests that the foreign CTL epitopes are efficiently presented by the chimeric *gag*–V3 VLPs. In whole, the consensus V3 domain sequence efficiently elicited a broadly cross-reactive CTL response that covers the clade B of HIV-1 which represents most North American isolates. Very recently it has been demonstrated that clade B-based canarypox vaccines can elicit broad CTL reactivities capable of recognizing viruses belonging to genetically diverse HIV-1 clades (Ferrari *et al.,* 1997).

Although the molecular mechanism of CTL induction is not clear, the fact that *gag*–V3B and *gag*–V3C induce class I-restricted T cell reflect that the CTL epitope presented by chimeric particles is able to enter the antigen processing pathway that leads to the association with class I MHC molecules. Therefore, the incorporation of multiple V3 sequences into *gag* particles is an efficient method for the presentation of exogenous antigens and induction of a dominant CTL response. The entry of exogenous protein to the endogenous class I processing pathway has been reported in some viral polypeptides (Ahlers *et al.,* 1996; Sauzet *et al.,* 1995; Zamorano *et al.,* 1994; Hsu *et al.,* 1995) and particulate structures such as yeast-derived Ty virus-like particle (Layton *et al.,* 1996), chimeric HIV-1 virus like particles (Wagner *et al.,* 1996), hepatitis B virus surface antigen particles (Schirmbeck *et al.,* 1994), and biodegradable microparticles as a delivery for measles viruse CTL epitopes (Partidos *et al.,* 1996). The exogenous viral proteins and virus-like particles are processed for CTL induction have also been proven in dendritic cells (Bachmann *et al.,* 1996). A few years ago, Hilleman (1995) compared the immunology and pathogenesis of AIDS, hepatitis B, and measles for developing a HIV vaccine and indicated that it was a failure in the past to emphasize the use of *gag* gene-encoded antigens in vaccines that may be more conserved antigenically and critical to protective CTL responses, as in hepatitis B. Virus-like particles as vaccine candidates offer the advantage of being a safe and economic comparison to inactivated or attenuated whole viruses. The noninfectious VLPs carrying the considered antigenic domain could be expressed in large quantities and is easy for purification. This system allows the selective expression of a defined region of HIV protein in an immunoprominent epitope and therefore enables a specific immune response to be induced. The ability of fusing a large epitopes onto a noninfectious *gag* particles opens up another way of designing vaccines against HIV infection.

## MATERIALS AND METHODS

### Plasmid construction

To construct hybrid *gag*–multiple V3 genes, the plasmid pUC19 containing the HIV2-$gag_{425}$ gene was employed as a carrier as previously described (Luo *et al.,* 1992). Synthetic multiple V3 loop sequences were inserted into the *Bgl*II site located at the C terminus of *gag*

gene to extend the *gag* reading frame and resulted in the generation of chimeric *gag*–V3 genes. Figure 1 shows the three constructs of the plasmids used in this study and indicates the inserted *env* gene sequences. All inserted sequences were confirmed by dideoxy DNA sequencing of the double-stranded DNA, using the Sequenase kit (United States Biochemical Corp).

### Production of recombinant baculovirus

The generation of recombinant baculovirus was carried out by cotransfecting SF21 cells with linearized wild type BacPAK6 viral DNA and transfer vector pBacPAK1 DNA by using liposome-mediated gene transfer with lipofectin technique provided in the BacPAK baculovirus expression system kit (Clontech Laboratories, Inc.) according to the manufacturer's instruction. A few viral plaques were picked and recombinant viruses were verified by Western blot ((Luo *et al.,* 1992). The newly isolated recombinant virus was purified by further consecutive plaque assay and used to produce a virus stock of $2 \times 10^8$ PFU/ml.

### Purification of chimeric *gag* particles

SF21 cells were infected with recombinant baculovirus at a multiplicity of infection of 5 PFU/cell and incubated at 27°C for 72 h. The cell culture supernatant was collected after centrifugation at 1000 *g* for 20 min. Sedimentable particles in the culture supernatant were collected by ultracentrifugation at 80,000 *g* for 1 h using a SW 28 rotor and resuspended in PBS containing 0.1% Tween 20 and 10 μg/ml aprotinin. The chimeric *gag* particle band was collected from a 20–60% step sucrose gradient after centrifugation at 100,000 *g* for 4 h in a SW41 rotor and diluted at least 10-fold with PBS and repelleted by centrifugation at 80,000 *g* for 1 h in a SW28 rotor (Luo *et al.,* 1992, 1994). The pellet was gently resuspended in phosphate-buffered saline (PBS) and used for further analyses including electron microscopy.

### Transmission electron microscopy

The electron microscopy was carried out as described previously (Luo *et al.,* 1994). Briefly, the samples were placed onto a carbon grid and then stained with 1% uranyl acetate and examined in a Philip 300 transmission electron microscope.

### Protein expression and immunoprecipitation

SF21 cells were infected with either wild type BacPAK6 or recombinant baculovirus at a multiplicity of infection of 5 PFU/cell and incubated at 27°C. After appropriate incubation time, cells were harvested and washed twice with PBS. Whole cell lysates were prepared by resuspending the cell pellets in water and adding an equal volume of 2× dissociation buffer (10% β-mercaptoethanol, 10% sodium dodeayl sulfate (SDS), 25% glycerol, 100 mM Tris–HCl, pH 6.8, and 0.04% bromophenol blue). Cell lysates were analyzed in 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and the protein bands were visualized by staining with Coomassie blue and confirmed by using $ECL_{TM}$ Western blotting kit (Amersham Life Science). For the analysis of antigenicity of chimeric VLPs, pulse-labeling of infected SF21 cells was carried out as previously described (Li *et al.,* 1993, 1994). Briefly, at 24 h (p.i.), the infected SF21 cells were incubated in methionine-free Grace's medium for 30 min and pulse-labeled for 1 h using [$^{35}$S]methionine (250 μCi/ml). Cells were lysed in an extraction buffer (50 mM Tris–HCl, pH 8.0, 1% NP40, 150 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride). Anti-V3 antiserum was added to the clarified cell lysate and incubated at 4°C for 12 h. Immune complexes were recovered with protein A–Sepharose CL-4B beads and washed three times with RIPA buffer (50 mM Tris–HCl, pH 8.0, 1% NP40, 150 mM NaCl, 1% deoxycholate, 0.1% SDS, and 1 mM EDTA). Protein samples were analyzed by 12% SDS–PAGE and visualized by fluorography.

### Generation and measurement of antiserum to chimeric *gag–env* fusion protein

Purified *gag*–V3 chimeric particles containing multiple neutralizing and CTL epitopes of HIV-1 were used to generate antiserum. Six New Zealand white rabbits were immunized with 30 μg of purified chimeric *gag*–V3 particles by four intramuscular injections at 1-month intervals. The immunogens were formulated with complete Freund's adjuvant for primary injection and with incomplete Freund's adjuvant for boosting at 1:1 ratio. Serum samples were taken before the injections and 2 weeks after each injection.

The anti-V3 and anti-*gag* responses were evaluated in a solid-phase enzyme-linked immunosorbent assay (ELISA) by using the *gag*–V3A, *gag*–V3B, and *gag*–V3C-related synthetic V3 domain peptides (see Fig. 1), the purified chimeric *gag*–V3, or the *gag* particles. All samples in carbonate–bicarbonate, pH 9.6, were coated on microtiter plates (Microtest III flexible assay plate, Becton Dickinson, Oxnard, CA) at a concentration of 100 ng/well at 37°C overnight. After the plates were washed three times with PBS containing 0.5% Tween 20, the wells were blocked with 5% nonfat dry milk powder dissolved in PBS at 37°C for 1 h. After washing three more times, the plates were incubated with a serial twofold dilution of the various antisera at 37°C for 1 h and processed with the respective secondary antibodies conjugate to horseradish peroxidase diluted in 1:3000 (Bio-Rad) for another hour at 37°C. The microtitre plates were washed five times between incubations, the unbounded conjugate was removed and the bounded enzyme was detected by the addition of 100 μl of a 2,2″-

azino bis[3-ethylbenzthiazolin-6-sulfonic acid] and $H_2O_2$ solution, a commercially available kit (Peroxidase Substrate Kit, Bio-Rad, Hercules, CA), according to the manufacturer's instructions. After 10 min of color development, the absorbance was read in a Bio-Rad Model 550 microplate reader at 415 nm. Virus neutralization assay was carried out as described previously (Luo *et al.,* 1992; Wagner *et al.,* 1996).

### Preparation of liposomes

Multilamellar vesicles (MLV) were prepared by vortexing. Lipids (phosphatidylethanolamine-β-oleoyl-α-palmitoyl:cholesteryl hemisuccinate = 7:3 mole ratio) were dissolved in 1 ml chloroform and dried under a stream of nitrogen gas to form a thin film on the wall of a glass tube. A PBS buffer solution containing protein antigen (*gag*–V3B and *gag*–V3C) was introduced into the glass vial and vortexed vigorously. Freezing–thawing vesicles (FTV) were prepared as described by Zhou *et al.* (1991). Briefly, small unilamellar vesicles (SUV) were produced by sonicating MLV and further incubated at room temperature for 1 h. After that, the SUV was briefly sonicated and then rapidly frozen in liquid nitrogen and slowly thawed at room temperature. The freezing–thawing process was repeated for a total of four times.

### Cytotoxic T-lymphocyte assay

Female Balb/C mice (5–6 weeks old) were immunized by intramuscular injection with either 50 μg of *gag*–V3C or *gag*–V3B chimeric particles in a 1:20 (protein:lipid) ratio with liposome entrapped. The boosting was performed at day 7 after the first immunization by the same route. The spleens were removed from immunized mice 4 weeks after the final immunization, and spleen-cell suspensions were prepared and pooled from two mice per group. The pooled cells were divided into four aliquots and restimulated *in vitro* for 7 days with individual V3 peptides (4 μg/ml) representing four HIV-1 isolates: $V3_{IIIB}$ (RIQRGPGRAFVTIGK), $V3_{MN}$ (HIGPGRAFYTTKN), $V3_{RF}$ (TKGPGRVIYATGQ), and $V3_{SF2}$ (YIGPGRAFHTTGR). For preparation of peptide-stimulated target cells p815 [a MHC-matched (H-$2^d$) mastocytoma cell line], the p815 cells ($1 \times 10^6$/ml) were preincubated individually with 20 μg of V3 peptides representing IIIB, MN, RF, or SF2 strains of HIV-1 clade B for 16–17 h and labeled with $^{51}Cr$ for another 2 h at 37°C. Control target cells were incubated with either medium alone or with a oligopeptides representing lymphocytic choriomeningitis virus (LCMV) nucleocapsid protein NP, 119–127: (PQASGVYMG).

The CTL activity against peptide-stimulated target cells was determined in a standard 6-h $^{51}Cr$-release assay. Aliquots of $5 \times 10^5$, $2 \times 10^5$, and $1 \times 10^5$ effector cells per well were mixed with $2 \times 10^4$ $^{51}Cr$-labeled target cells for 6 h in 96-well microtiter plates, and then 100 μl of supernatant was collected for $^{51}Cr$-release assay. The percentage-specific $^{51}Cr$-release was calculated as 100 × (sample release − spontaneous release)/(maximum release − spontaneous release). Spontaneous release was determined from target cells incubated with culture medium and was less than 15% in all experiments. The maximum release was determined from target cells lysed by 2% Triton X-100. Each E/T ratio was tested in triplicate and repeated at least twice.

### Depletion of $CD4^+$ or $CD8^+$ cells

$CD4^+$ cell- or $CD8^+$ cell-depleted populations were obtained by incubating restimulated splenocytes with anti-CD4 or anti-CD8 monoclonal antibodies, respectively. Resulting cells were then tested for their capacity to lyse MHC-matched p815 target cells that were pretreated with $V3_{IIIB}$ peptide. In this experiment, CTL effectors and target cells were mixed at various E:T ratios of 25:1, 10:1, and 5:1. The culture supernatant of $^{51}Cr$-labeled cells were harvested and determined by $^{51}Cr$ release assay.

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